Variants in the 3'UTR of the ovine Acetyl-Coenzyme A Acyltranferase 2 gene are associated with dairy traits and exhibit differential allelic expression

Miltiadou, D; Hager-Theodorides, AL; Symeou, S; Constantinou, C; Psifidi, A; Banos, G; Tzamaloukas, O

Published in:
Journal of Dairy Science

DOI:
10.3168/jds.2016-12326

First published: 16/06/2017

Document Version
Publisher's PDF, also known as Version of record

Interpretive summary

Variants in the 3'UTR of the ovine Acetyl-Coenzyme A Acyltransferase 2 gene are associated with dairy traits and exhibit differential allelic expression. *By Miltiadou et al.* The present study shows that the ovine *ACAA2* gene is associated with milk production, milk protein percentage and milk fat yield. Additionally, we demonstrated that the mRNA expression in homozygous animals for each allele of the *ACAA2* gene differs by several orders of magnitude in hepatic and mammary gland tissues and the *ACAA2* variants are expressed at different levels in the udder of heterozygous animals, suggesting the existence of regulatory variation within the ovine *ACAA2* gene. These findings support the hypothesis that the *ACAA2* gene is a functional candidate affecting dairy traits in sheep.

28 **Variants in the 3'UTR of the ovine Acetyl-Coenzyme A Acyltransferase 2 gene**
29 **are associated with dairy traits and exhibit differential allelic expression.**
30
31 D. Miltiadou[*1,2], A. L. Hager-Theodorides[†1], S. Symeou[*], C. Constantinou[*], A.
32 Psifidi[‡], G. Banos[‡,§] and O. Tzamaloukas[*]
33
34 [*]Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus
35 University of Technology, Lemesos, PO Box 50329, Cyprus
36 [†]Department of Animal Science, Agricultural University of Athens, Greece
37 [‡]Roslin Institute, University of Edinburgh, EH25 9RG Midlothian, UK
38 [§]Scotland's Rural College, EH25 9RG Midlothian, UK
39
40 [1] Both authors contributed equally to this work.
41 [2] Corresponding author: Department of Agricultural Sciences, Biotechnology and
42 Food Science, Cyprus University of Technology, PO Box 50329, Limassol, Cyprus,
43 tel: +357 25002590, fax: +357 25002767, Despoina.Miltiadou@cut.ac.cy
44
45
46
47
48
49
50
51

ABSTRACT

The Acetyl-CoA acyltransferase 2 (*ACAA2*) gene encodes an enzyme of the thiolase family that is involved in mitochondrial fatty acid elongation and degradation by catalyzing the last step of the respective β-oxidation pathway. The increased energy needs for gluconeogenesis and triglyceride synthesis during lactation are met primarily by increased fatty acid oxidation. Therefore, the *ACAA2* enzyme plays an important role in the supply of energy and carbon substrates for lactation and may thus affect milk production traits. This study investigates the association of the *ACAA2* gene with important sheep traits and the putative functional involvement of this gene in dairy traits. A single nucleotide substitution (SNP), a T to C transition located in the 3' untranslated region (UTR) of the *ACAA2* gene, was used in mixed model association analysis with milk yield, milk protein yield and percentage, milk fat yield and percentage, and litter size at birth. The SNP was significantly associated with total lactation production and milk protein percentage, with respective additive effects of 6.81 ± 2.95 kg and -0.05 ± 0.02 %. Additionally, a significant dominance effect of 0.46 ± 0.21 was detected for milk fat yield. Homozygous TT and heterozygous CT animals exhibited higher milk yield compared to homozygous CC animals, while the latter exhibited increased milk protein percentage. Expression analysis from age, lactation and parity matched female sheep showed that mRNA expression of the *ACAA2* gene from TT animals was 2.8 and 11.8 fold higher in liver and mammary gland, respectively. In addition, by developing an allelic expression imbalance (AEI) assay, it was estimated that the T allele was expressed at an average of 18% more compared to the C allele in the udder of randomly selected ewes. We demonstrated for the first time that the variants in the 3'UTR of the ovine *ACAA2* gene are differentially expressed in homozygous ewes of each allele and exhibit AEI

77 within heterozygotes in a tissue specific manner, supporting the existence of cis-
78 regulatory DNA variation in the ovine *ACAA2* gene. This is the first study reporting
79 differential allelic imbalance expression of a candidate gene associated with milk
80 production traits in dairy sheep.
81
82 Key words (up to 4): *ACAA2* association, *3' UTR* Cis-acting SNP, gene expression,
83 dairy sheep

INTRODUCTION

Milk yield represents more than two thirds of the total income of the dairy sheep industry (Carta et al., 2009) and therefore the improvement of milk production is the most important breeding objective. In Mediterranean countries, however, most of the ovine milk produced is used for the production of cheese commercialized as products of protected designation of origin (PDO) and other quality labels (Arranz and Gutierrez-Gil, 2012). Thus, farmers' income is additionally determined by total solids that affect cheese yield (De Rancourt et al., 2006) and, therefore, increased milk fat and protein content is also highly desirable from an economic perspective (Ramon *et al.*, 2010). Although traditional breeding programs have achieved appreciable genetic gains mainly for milk yield, application of selection schemes assisted by molecular information could expedite improvement (Carta et al., 2009). Moreover, marker assisted selection could be of special interest for dairy sheep, due to the great regional diversity of breeds, funding limitations, organizational difficulties (Arranz and Gutierrez-Gil, 2012) and small population sizes (Garcia-Gamez et al., 2012).

To date, there are few reports of genome-wide association studies (GWAS) and genome scans based on linkage mapping that detect quantitative trait loci (QTL) and quantitative trait mutations (QTM) for ovine dairy traits (reviewed by Arranz and Gutierrez-Gil, 2012; Garcia-Gamez et al., 2012; 2013; Gutierrez-Gil et al., 2014). In a whole genome QTL study performed in Churra ewes, Gutierrez-Gil et al. (2009) detected suggestive QTL for milk, fat and protein yields, mapped in a region of the ovine chromosome 23 (OAR23) harbouring the Acetyl-CoA acyltransferase 2 (*ACAA2)* gene. The *ACAA2* gene encodes an enzyme of the thiolase family, also known as 3-oxoacyl-CoA thiolase or mitochondrial 3-ketoacyl-CoA thiolase. The *ACAA2* enzyme catalyzes the last step in mitochondrial fatty acid β-oxidation, thus

playing a central role in the supply of energy for the animal (Bartlett and Eaton, 2004). Therefore, due to the chromosomal location of the ovine *ACAA2* gene in relation to the QTL described by Gutierrez-Gil et al., (2009) and its functional role in lipid metabolism, it was regarded as a putative functional and positional candidate gene that may affect milk yield and composition.

Genes encoding enzymes of the thiolase family have been correlated with production traits in other livestock species. Single nucleotide polymorphisms (SNPs) detected in the porcine *ACAA2* gene are reported to be associated with daily weight gain and loin muscle area (Li HD, 2008). An important paralog of the *ACAA2* gene, the acetyl-CoA acetyltransferase 2 (*ACAT2*) gene has been associated with production and fertility traits (milk protein content, productive life and conception and pregnancy rates) in Holstein cattle (Cochran et al., 2013), while SNPs within the swine *ACAT2* gene were suggested to influence the metabolic functions of the corresponding enzyme and thus may affect growth performance (Sodhi et al., 2014).

Our previous study showed that the entire mRNA (coding and untranslated regions) of the ovine *ACAA2* gene is monomorphic in Chios sheep, one of the most productive and extensively used breeds in Greece and Cyprus (Chatziplis et al., 2012), with the exception of a SNP (HM537015:g.2982T>C) located in the 3' untranslated region (*UTR*) of the gene (Orford et al, 2012). The SNP was significantly associated with milk yield, at first lactation and across first to third lactations in Chios sheep. Animals from a closed nucleus research flock at the Agricultural Research Institute of Cyprus, carrying the g.2982TT or g.2982CT genotype had significantly higher milk yield than those with the g.2982CC genotype and the g.2982T>C SNP explained 10% of the additive genetic variance for milk yield when data up to third lactation from a single flock were analysed (Orford et al., 2012).

It is well established that *UTRs* contain motifs involved in posttranscriptional regulation of gene expression (Xie et al., 2005) that may lead to differential expression of alleles associated with phenotypic diversity of production traits (Clop et al., 2006; Khatib et al., 2007; Sugimoto et al., 2015). Studies in humans (Yan et al., 2002, Bray et al., 2003), mice (Cowles et al., 2002), cattle (Khatib et al., 2007, Olbromski et al., 2013) and pig (Muráni et al., 2009) have shown that alleles of non-imprinted genes are not expressed equally at the mRNA level in heterozygous animals, a phenomenon called allelic expression imbalance (AEI). AEI is the outcome of the presence of at least one cis regulatory element in the regulatory sequences of a gene (Campbell et al., 2008) and is therefore one of the possible mechanisms underlying the effect of causative genetic variations that are not located on the translated region of a gene.

The objective of the current study was to provide novel insights into the association of the ovine *ACAA2* gene with important sheep traits. Firstly, we performed an association analysis of the previously identified g.2982T>C SNP with milk yield, fat and protein contents, fat and protein yields and litter size, in a population of Chios sheep from all available farms keeping production records in Cyprus. Upon confirmation of the association of the SNP with total milk production and additional detection of association with milk protein percentage, we tested the hypothesis that the *ACAA2* is a putative functional gene affecting dairy traits, by comparing the expression of the gene in different genotypes and by developing an assay to test the presence of AEI.

MATERIALS AND METHODS

Animals and phenotypic data

159 Data were collected from 742 purebred Chios ewes from five farms. Those were
160 one governmental farm (Orites, Cyprus) and the only 4 commercial farms in Cyprus
161 keeping phenotypic records of purebred Chios sheep according to the regulations of
162 the International Committee for Animal Recording (ICAR).

163 For all animals, individual records included month of lambing, year of lambing,
164 lactation number and age of lambing. Phenotypic data were obtained for lambing
165 years between 2009 and 2016 and included 1,514 individual records of 742 milking
166 ewes; this data included total lactation milk yield, 1,242 observations for litter size at
167 birth, 1,203 measurements of milk fat content and yield and 615 measurements of
168 milk protein content and yield, respectively. Total lactation yield was calculated for
169 each animal with the Fleischmann method with monthly tests on actual yields (sum of
170 *am* and *pm* records, ICAR 2014). Milk samples were obtained for fat and protein
171 analysis by combined thermo-optical procedures (LactoStar 3510, Funke Gerber,
172 Berlin, Germany), previously calibrated for protein with the Lowry protein assay and
173 for fat with the method 989.05 (AOAC International, 2005).

174

175 ***DNA extraction and SNP genotyping***

176 Whole blood samples were obtained from all 742 ewes for DNA extraction and
177 genotyping of the g.2982T>C SNP of the *ACAA2* gene. Genomic DNA was isolated
178 from all samples using the Genomic DNA Blood kit (Macherey-Nagel), according to
179 the manufacturer's instructions, and DNA quality and quantity were estimated by UV
180 absorption at 260 and 280nm. Genotyping of the Chios ewes was performed by a cost
181 effective direct DNA sequencing protocol (Miltiadou et al., 2017), with primers
182 amplifying the 10^{th} exon of the gene, using conditions previously described (Orford et
183 al., 2012).

184

185 ***Mixed model association analysis***

186 Each trait was analyzed separately with the following mixed linear model:

187 $$Y_{jklmn} = \mu + F_i + YS_j + L_k + b_1 age + b_2 dur + G_l + A_m + e_{ijklmn} \quad (1)$$

188 where:

189 Y = lactation milk yield, fat yield/percentage, protein yield/percentage or litter size
190 record *n* of animal *m*

191 μ = overall population mean for the trait

192 F = fixed effect of flock *i* (1-5)

193 YS = fixed effect of year (2009-2016) by season (1-2) of lambing interaction *j*

194 L = fixed effect of number of lactation *k* (1-4)

195 b_1 = linear regression on age at lambing (age)

196 b_2 = linear regression on lactation duration (dur); yield traits only

197 G = fixed effect of genotype l in the g.2982T>C locus (1-3; CC, CT, TT)

198 A = random effect of animal *m*

199 e = random residual effect

200

201 All lactation records were analyzed for all traits. In a separate analysis, only first
202 lactation records were considered for milk yield. In the latter case, the fixed effect of
203 number of lactation and the random animal effect were removed from the model.

204 In all cases, predicted trait values for each SNP genotype and respective standard
205 errors were derived; these values were reflective of the marginal genotypic effect on
206 each trait adjusted for all other effects fitted in the model. The predicted trait values
207 were used to estimate additive and dominance SNP effects on traits, and the

proportion of phenotypic variance for each trait accounted for by the SNP locus. The equations used were:

Additive effect, $a = (TT - CC) / 2$;

Dominance effect, $d = CT - [(TT + CC) / 2]$,

Percentage of phenotypic variance (V_P) due to SNP = $100 * [2pq(a + d(q-p))^2] / V_P$,

where TT, CC, CT are the predicted trait values for each genotype class and p, q are the allele frequencies at the SNP locus. Variance components were estimated with model (1) after excluding the genotype effect. All statistical analyses were conducted with the ASReml3 software (Gilmour et al., 2009).

Since multiple traits were analyzed, we assessed the number of independent tests in order to adjust for multiple testing. For this reason, we conducted multivariate analyses based on model (1) after excluding the genotype effect to assess the correlation among traits. Very high correlations were found among the three production traits (milk, fat, protein yield; 0.89-0.99), and between the two milk concentration traits (fat and protein percentage; 0.75). Correlations between the two groups of traits were near zero. Correlations of these two groups with litter size ranged from 0.05 to 0.19. Consequently, we regarded three separate trait groups (production, concentration and litter size) that corresponded to three distinct, independent hypothesis tests. This was confirmed with a Principal Component Analysis of the studied traits (Supplementary Figure 1). Subsequently, a Bonferroni adjustment for multiple tests was implemented based on the Holm-Bonferroni method (Holm 1979). This method works sequentially testing first the lowest nominal P-value against the threshold value of 0.05/n, where n is the number of independent hypotheses tests (three in the present study). If this hypothesis is rejected, the next lowest nominal P-value is compared to 0.05/(n-1) and so on, until a hypothesis is not

rejected (Holm, 1979). Supplementary Table 1 includes the corresponding P-value thresholds in the case of three independent tests.

Animals and tissue sampling for expression analysis

Blood samples were collected from 161 first parity ewes from the biggest commercial farm in Cyprus, allowing the selection of age, parity and lactation stage matched animals. Based on the methodology described in our previous work (Orford et al., 2012), we identified animals with the genotypes g.2982TT, g.2982CT and g.2982CC, and chose 3 ewes from each genotype for subsequent biopsies and RNA extraction. All 9 selected ewes were first parity, 15-month old and had given birth to two lambs each.

Mammary and liver biopsies were obtained under anaesthesia, by a professional veterinarian, 42 ± 2 days after lambing, a week after weaning. Liver parenchyma was sampled via puncture biopsy using ultrasonography for the selection of the appropriate biopsy site and for avoiding large vessels and other organs. Udder biopsies were taken from either the left or the right rear gland. The biopsy site was carefully selected to avoid large subcutaneous blood vessels. Preparation of the site involved shaving and washing with dilute betadine solution followed by sanitizing with ethanol (70%). Ewes were given intravenous xylazine before anesthetizing the biopsy site by subcutaneous injection of lidocaine hydrochloride. An incision was made (~0.5-1.0 cm) on the outside of the quarter using a scalpel blade (size 22). A Bard® Magnum® core biopsy instrument (Bard Peripheral Vascular, Inc., Tempe, AZ) with a Bard® Magnum® core tissue biopsy needle (MN1210, 12G × 10 cm) was used.

In addition, liver and udder *ex vivo* tissue samples were obtained from 16 randomly selected Chios ewes from an abattoir straight after slaughtering, under sterile

conditions. Although tissue samples obtained from the abattoir were at different and unknown parities and stages of lactation, they were taken to increase the number of heterozygous samples for allelic expression imbalance analysis, without increasing the veterinary costs required for biopsies.

All samples were snap frozen in liquid N_2 immediately after the tissue was obtained under sterile conditions and stored at −80°C until RNA extraction.

Animal sampling and handling and all procedures in this study were carried out in strict accordance with the national legislation for animal welfare 46I/94 and no animals were sacrificed for the purposes of this study.

DNA, total RNA extraction and reverse transcription

Frozen tissue biopsies and ex vivo samples were homogenized using a mortar and pestle constantly covered under liquid N_2 and aliquoted in tubes containing ~20 mg each. DNA was extracted using the Genomic DNA Nucleospin Tissue kit (Macherey-Nagel, Germany), while ~20 mg of frozen homogenized tissue was subjected to RNA extraction using the RNA isolation Nucleospin RNA kit (Macherey-Nagel, Germany), according to manufacturer's instructions. Before reverse transcription, extracted RNA was incubated with 2U/µl rDNAase (Macherey-Nagel, Germany) for 10 min at 37°C to eliminate putative contaminating genomic DNA and then purified using the RNA clean up Nucleospin RNA Clean up XS kit (Macherey-Nagel, Germany). The concentration and quality of DNA and RNA for all samples was measured using a Nanodrop 1000 UV/VIS spectrophotometer (Thermoscientific Nanodrop Technologies LLC, USA). The RNA integrity was assessed by electrophoretic analysis of the 28S and 18S rRNA subunits.

Complementary DNA (cDNA) was synthesized from 0.5 µg RNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), using random hexamer primers following the manufacturer's recommendations in a final volume of 20 µl. The lack of genomic DNA contamination was verified by a PCR amplification using primers amplifying intron 2 of the prolactin gene (*PRL*), as described by Orford et al. (2010) and by including a control without reverse transcriptase during cDNA synthesis of each sample. These controls were used for conventional PCR amplification of the *ACAA2* exon 10, as we have previously described (Orford et al., 2012) and for subsequent real time PCR (RT-PCR) and verified lack of genomic contamination.

ACAA2 mRNA expression

ACAA2 relative expression in liver and udder was assessed by quantitative Reverse Transcriptase PCR (qRT-PCR). *ACAA2* qRT-PCR was performed with the primers used for genotyping as described above and expression levels were normalized by the expression of reference genes, ovine β-actin (*ACTB*) for liver samples and ovine β2 microglobulin (*ovB2M*) for udder samples, utilizing primers: ovACTB Forward (GCAAAGACCTCTACGCCAAC) and ovACTB Reverse (TGATCTTGATCTTCATCGTGCT) (Sari et al., 2009) and for ovB2M Forward (CTGTCGCTGTCTGGACTGG) and ovB2M Reverse TTTCCATCTTCTGGCGGGTG (designed using the NCBI/Primer-BLAST tool). For each gene and cDNA sample qRT-PCR reactions were performed in triplicates. Reactions of no template negative control and 5 point 1:5 serial dilutions of reference cDNA, to obtain the reaction standard curves and calculate amplification efficiencies, were also performed in triplicates. Reactions for *ACAA2* expression were performed

in a final volume of 20µl containing 1× KAPA SYBR fast qPCR Master Mix (Kapa Biosystems), 100 nM of each of *ACAA2*_SNP Forward and Reverse primers, 1xROX low and cDNA. Reactions for *ACTB* and *B2M* were performed in a final volume of 10µl containing 1× KAPA SYBR fast qPCR Master Mix (Kapa Biosystems), 200nM of each of the ovACTB or ovB2M Forward and Reverse primers, 1xROX low and cDNA. Cycling conditions included a 3 min initial denaturation step at 95°C followed by 40 cycles of: (i) denaturation for 3 sec at 95°C and primer annealing and template extension for 30 sec at 60°C, for *ACAA2* (ii) denaturation for 3 sec at 95°C, primer annealing at 62°C for 20 sec and template extension at 72°C for 30 sec, for A*CTB* and (iii) denaturation for 3 sec at 95°C, primer annealing at 64°C for 15 sec and template extension at 72°C for 30 sec, for *B2M*. For all three genes, a melting curve analysis was performed following the amplification cycles consisting of 15 s at 95°C, 30 s at the respective annealing temperature for each gene and continuous heating and data collection up to 95°C at a rate of 1% temperature increase per 30 seconds, to evaluate specificity of the amplification. Reactions were optimized for amplification efficiency to be between 85-95% and linear standard curve fit (r^2) to be greater than 0.990 for all genes. Raw data were analyzed with the 7500 software v2.3 (Applied Biosystems, USA), and mean Ct values and PCR reaction efficiencies were exported. The normalized expression (nQ) of *ACAA2* was calculated according to the delta-Ct method (Livak and Schmittgen, 2001), i.e. $nQ = E_{ACAA2}\text{^}(min_{ACAA2\_Ct}\text{-}sample_{ACAA2\_Ct})/E_{ref}\text{^}(min_{ref\_Ct}\text{-}sample_{ref\_Ct})$, where E is the PCR efficiency for a given gene (values are greater than 1 and 2 corresponds to ideal doubling of templates per cycle, 100% = 2) estimated based on standard cDNA dilution series reactions, min_{gene_Ct} is the minimum Ct value among all samples for a given gene and $sample_{gene\_Ct}$ is the Ct value for a given gene and sample. To express *ACAA2* nQ as

fold change relative to nQ in CC genotype (nrQ) nQ for each sample was divided by mean nQ in CC samples. Data were then analyzed using the mixed models procedure of SAS software (SAS, 2005) with genotype as the fixed effect. Mean comparisons were performed with Tukey's adjustment with significance level set at 0.05.

Allelic Expression Imbalance (AEI) analysis

Sequencing of the *ACAA2* gene from genomic DNA and cDNA of each sample was initially used as a semi-quantitative approach to detect differential expression of the *ACAA2* alleles. RT-PCR products amplified from heterozygous individuals were sequenced and data were analyzed using Applied Biosystems' Sequencing Analysis (Applied Biosystems, USA). The SNP was identified by visually inspecting each base in sequencing traces. Allelic variation was estimated by comparing the proportions of the peak heights of the two alternative alleles of the SNP.

Subsequently, the detection of AEI was based on quantitative analysis of mRNA transcripts using a TaqMan probe qRT-PCR assay (Livak, 1999, Chen et al., 2008) in order to detect deviations from the null hypothesis expecting equimolar ratio between the two alleles in heterozygous samples. TaqMan qPCR reactions were performed in 10 µl reaction volumes containing 1× Type-it® Fast SNP Probe PCR Master Mix (Qiagen, USA), 400 nM of each PCR primer and 200 nM of each TaqMan probes (primer and probe sequences as described in the *DNA extraction and SNP genotyping* section), and liver or udder cDNA or gDNA from *ACAA2* heterozygous ewes. A 7500 Real Time PCR system (Applied Biosystems, USA) was used with a cycling profile that included a 5 min denaturation step at 95°C and 40 cycles of 15 sec at 95°C and 30 sec at 62°C.

Pooled genomic (g) DNA from three *ACAA2* heterozygous animals (genotype confirmed by several replications of genotyping reactions) was used as standard gDNA and 5 points of 1:4 serial dilutions were used to construct the standard curve for relative *ACAA2* T and C allele quantification (Fig. 1A-C). The T:C allele copy ratio is expected to equal 1 in all dilutions.

The quantitation relative to standard curve method was employed, using the homonymous option of the ABI 7500v2.3 software to extract the value for "normalized quantity" of T allele at each qPCR reaction, having set the C allele as the "endogenous control". The "normalized quantity" of the T allele was computed as the quantity of T allele divided by the quantity of C allele and thus corresponds to the ratio of T:C *ACAA2* allele quantity. T and C allele quantities in each qPCR reaction were computed from the respective Ct values and the linear prediction function for each of the *ACAA2* alleles, computed by the software based on standard gDNA dilution series. The quantity of T and C alleles in the standard gDNA was set at the same arbitrary value and thus T:C ratio equalled 1 at all points of the dilution series of the standard gDNA. The T:C ratio for each sample, i.e. the mean normalized quantity of the three replicates performed with each cDNA or gDNA, was divided by the average normalized quantity of the T allele in all the gDNA samples to obtain the corrected T:C ratio for each cDNA sample. Average corrected T:C ratio in the gDNA samples equalled 1 and ranged between 0.90-1.09. Standard gDNA dilution series reactions were performed in each qPCR run. In all runs, efficiency values for both T and C allele amplification were very similar (102.01% for T and 101.23% for C in run 1 and 102.66% for T and 102.74% for C in run 2). Linear standard curve r^2 values were 0.997 for T, 0.996 for C in run 1 and 0.998 for T and 0.998 for C in run 2.

Statistical analysis of the T:C ratios in cDNA or gDNA was performed using the mixed models procedure of SAS software (SAS, 2005) with tissue (liver or udder) as the fixed effect. Means are presented as ordinary means and se. Least square mean pairwise comparisons were performed with Tukey's adjustment with significance level set at 0.05.

RESULTS

Association of the ACAA2 gene with sheep traits

Allelic frequencies in the g.2982C/T SNP locus were 0.54 for the T allele and 0.46 for the C allele; genotypic frequencies were 0.27, 0.54 and 0.19 for TT, CT and CC, respectively. Genotypic frequencies were found to deviate from the Hardy-Weinberg equilibrium ($p = 0.019$).

Marginal predicted means for the three genotype classes for all production traits are presented in Table 1. After applying Holm-Bonferroni correction, the SNP was significantly associated with milk yield at first lactation ($P < 0.01$) and all lactations ($P < 0.025$), with respective additive effects of 10.61 ± 3.56 kg and 6.81 ± 2.95 and respective positive dominance effects of 13.02 ± 4.26 kg and 8.67 ± 3.53 (Table 2). Significant differences were found between the CC and CT genotype pairs ($P < 0.01$) and between the CC and TT classes ($P < 0.025$) at the g.2982T>C SNP locus for milk yield (Table 1). These results overall suggest a complete dominance effect at the locus, since heterozygous CT animals exhibit similar predicted mean values compared to homozygous TT animals. Based on the estimated allelic effects and the allele frequencies observed in the sample, it was estimated that the g.2982T>C SNP explained 2.25% and 0.62% of the total phenotypic variance for 1st lactation and for all lactations, respectively (Table 2). Overall, these results suggest a stronger

405 association in first lactation milk yield, whereas the effect was reduced in subsequent
406 lactations.

407 After applying Holm-Bonferroni correction, the SNP was also associated with
408 protein percentage ($P < 0.01$), with a significant additive effect of -0.05 ± 0.02 (Table
409 1). Pairwise contrasts between the predicted protein percentage values showed
410 significant differences between the genotype classes CC and CT ($P < 0.001$) and
411 between CC and TT ($P < 0.025$), with CC animals exhibiting higher protein
412 percentage, whereas differences between CT and TT were not significant (Table 1). It
413 was estimated that the SNP explained 1.68% of the phenotypic variance for protein
414 percentage (Table 1). Additionally, there was a dominance effect of 0.46 ± 0.21 (p =
415 0.027) and 0.41 ± 0.21 (p = 0.048) of the SNP on fat and protein yields, respectively.
416 Homozygous TT and heterozygous CT animals exhibited significantly higher milk fat
417 yield compared to homozygous CC ewes ($P < 0.025$ and $P < 0.001$, respectively). No
418 significant associations of the SNP genotype with fat percentage, protein yield or litter
419 size at birth were found.
420
421 ***mRNA expression analysis***

422 The expression of *ACAA2* was found to be significantly increased in TT compared
423 to CC udder and liver biopsies from age matched, at the same lactation stage and
424 parity ewes that gave birth to two lambs each. In particular, homozygous TT animals
425 showed a 2.8 fold increase in mRNA expression levels compared to homozygous CC
426 animals in liver ($P < 0.05$), while TT ewes exhibited an 11.8 fold increase in mRNA
427 of the *ACAA2* gene in the udder compared to CC ewes ($P < 0.05$) (Fig. 2A). *ACAA2*
428 mRNA expression of heterozygous animals showed a tendency for reduction
429 compared to homozygous TT animals only in liver (p = 0.07), while it did not

significantly differ from homozygous CC animals. Heterozygous mRNA expression in the udder exhibited a very high standard deviation (Fig. 2A) and did not differ significantly from homozygote expression.

The expression of *ACAA2* was also assessed in liver and udder *ex vivo* samples from 1 CC, 10 CT and 5 TT ewes (Fig. 2B). These ewes were selected at random and were not age matched nor at the same milking season or lactation stage. Expression of *ACAA2* was found to be significantly increased in the liver compared to the udder ($P < 0.0001$), but no significant differences were observed between CT and TT ewes in either the liver or the udder ($P > 0.05$). Since only one ewe from the slaughterhouse was genotyped as CC, it was not possible to make comparisons between homozygotes.

Allelic expression imbalance analysis (AEI)

To test whether the differences observed between homozygous TT and CC animals, was due to AEI between the T and C alleles, the transcription of T and C alleles was quantified in liver and udder of heterozygous individuals. Sequencing of genomic DNA and cDNA from 12 heterozygous samples (3 from biopsies and 9 from slaughterhouse) was initially performed to evaluate semi-quantitatively whether there was allelic expression imbalance. The allele specific expression levels were evaluated by comparing the peak heights of C and T in cDNA samples from heterozygous individuals compared to the peak heights of the respective genomic DNA. Although in all genomic DNA samples from heterozygotes, peak heights of both nucleotides at the SNP position were tangential, suggesting no preferential amplification, peak heights from heterozygous cDNA samples were different, with the T allele exhibiting a higher peak compared to the C allele (Fig. 3).

To further confirm the initial results from the semi-quantitative method consistent with AEI in *ACAA2* gene, we developed an AEI assay using TaqMan probes to estimate the T:C transcription ratio in cDNA samples corrected by the mean T:C ratio in gDNA samples and ultimately test for deviations from the expected ratio of 1 in the absence of AEI. Results of the assay for the control gDNA reactions showed that observed T:C ratios were, as expected, very close to 1 (average non-corrected ratio was 1.052). *ACAA2* AEI was observed in individual ewes in both the liver and the udder (Fig. 4). The average corrected T:C ratio in the liver cDNA was 1.026, not significantly different from the average corrected gDNA ratio (mean = 1, p = 0.67). On the contrary, corrected T:C ratio of transcription in the udder of heterozygous animals was 1.18, significantly increased compared to both gDNA and liver cDNA mean corrected T:C ratios (p = 0.0054 and 0.0134 respectively). Therefore, we observed a moderate and tissue specific imbalance in the allelic expression of the two *ACAA2* alleles in favour of allele T in the udder, but not in the liver of heterozygous animals.

DISCUSSION

The study confirmed the previously observed association of the HM537015:g.2982T>C *ACAA2* SNP with milk yield in an extended population of Chios sheep from multiple flocks and showed that the g.2982T>C SNP of the *ACAA2* gene is also associated with milk protein percentage and milk fat yield. Investigation of the expression of the *ACAA2* gene from the three different genotypes and allelic expression imbalance analysis (AEI) of heterozygous samples supported the hypothesis that *ACAA2* is a functional gene affecting dairy traits. To the best of our

knowledge, this is the first study showing differential allelic imbalance expression of a candidate gene associated with milk production traits in dairy sheep.

Consistent with our previous study (Orford et al., 2012), the g.2982T>C SNP, was significantly associated with milk yield, with the T allele exhibiting positive additive and dominance effects, mainly attributed to first lactation production data. Confirmation of a previously detected association using a bigger and different data set strengthens the evidence for the observed association (Sasaki et al., 2013). The overall effects estimated in the present study, however, were lower compared to Orford et al., (2012), possibly due to the increased variation introduced from the use of multiple flocks managed in different ways, as a substantial fraction of the environmental variance for production traits is attributed to farm (Carta et al., 2009; Sasaki et al., 2013). In the current analysis, we adjusted for the systematic effect of the flock and, therefore, the estimates are more representative and indicative of the true effect of the gene in the population. The reasons why the effects are more important in the first lactation than in the rest of lactations are not clear at this stage and therefore further research is needed.

In the present study, additional evidence about the correlation of the *ACAA2* gene with important sheep traits is provided, as the g.2982T>C SNP was found to be significantly associated with milk protein percentage. Homozygous CC animals exhibited superior values for protein percentage compared to both homozygous TT and heterozygous CT animals, in contrast to their inferior values estimated for total milk yield (Table 1). This is consistent with the negative genetic correlation between those two traits (Bencini and Pulina, 1997; Fuertes et al., 1998). Since, protein yield was not significantly associated with this SNP, the decrease on protein content could be attributed to a dilution effect, due to the increase of milk yield (Emery, 1988).

Similarly, milk yield is known to be negatively correlated to fat content (Fuertes et al., 1998). However, although the marginal predicted mean for the fat content from CC ewes was higher compared to that of the other genotypes in the present study, the differences were not significant and the investigated SNP was not significantly associated with milk fat percentage (Tables 1 and 2), in consistence with the results of Orford et al. (2012). In agreement with other studies where fat is the most variable component of ovine milk (Othmane et al., 2002; Pulina et al., 2005), in the current study, standard errors for fat content were double compared to those for protein content. Therefore, low precision of data for fat content may be a reason for not detecting the association of the studied SNP with fat percentage. However, there is a significant association of the SNP with fat yield (Table 2). In addition, homozygous CC animals exhibit significantly lower fat yields, compared to heterozygous CT and homozygous TT animals (Table 1), that could be attributed to decreased milk yield with similar fat content.

Since fat and protein content are crucial for cheese-making, the potential use of the studied SNP in a selection scheme requires applied research to identify whether each additional unit of milk production compensates for the lower cheese yield due to lower protein percentage, based on farmer prices and cheese yield for a certain type of cheese. Using current prices in Cyprus for sheep milk and the values of each genotype in Table 1, the producer is going to get 1.012 euros extra per additional liter, whereas the price of the milk is reduced only by 0.012 (from 1.024 to 1.012) per liter due to the lower protein and fat content. Therefore, selecting for the T allele is expected to be beneficial. However, prior to incorporation of the SNP in any breeding program, negative effects of selection on other important traits, for instance fertility, need to be excluded. To facilitate further research, the SNP has been incorporated into the sheep

HD SNP chip developed by the International Sheep Genomics Consortium (ISGC) for functional studies (James Kijas, Csiro, Australia, personal communication).

Allele frequencies of the g.2982T>C polymorphism genotyped were similar to the previously observed (T:0.56; C:0.44; Orford et al., 2012). However, genotypic frequencies were found to deviate from the Hardy-Weinberg (H-W) equilibrium in the studied population, in contrast to the previously observed frequencies from a single experimental flock (Orford et al., 2012), due to higher frequencies of TT and CT ewes carrying the favourable for milk yield T allele. This could be possibly explained by the fact that farmers select animals mainly based on milk yield, due to low meat prices during the last decade, whereas the selection indices used at the experimental flock (Orford et al., 2012) combine the individual capacity of young stock for growth and milk production (Mavrogenis and Constantinou, 1991).

The candidate gene approach followed in the current study is an alternative to QTL and GWA studies that can be very powerful in the identification of loci even with small effects on the trait, if the candidate gene represents a true causative gene (Andersson, 2001). Otherwise, an association detected could occur due to linkage disequilibrium to linked or non-linked causative genes (Andersson, 2001). Therefore, the polymorphism in the *3' UTR* mRNA of the *ACAA2* gene that was found significantly associated with dairy traits, can be either the functional polymorphism to which the genetic variance can be attributed or can be in linkage disequilibrium with the functional polymorphism or both. Since the *ACAA2* g.2982T>C SNP is in the untraslated region of the gene and thus does not affect the protein produced, it may be a functional polymorphism playing a regulatory role, by causing alterations in the expression levels of the gene. Identification of regulatory variants requires studying two alleles of a gene under identical circumstances and comparing the expression

554 associated with each allele (Cowles et al., 2002). For that reason, the expression of the
555 *ACAA2* variants was investigated in first parity, age, lactation stage and litter size
556 matched ewes that were managed, fed and sampled uniformly to eliminate
557 confounding sampling and environmental effects. The practical difficulties of
558 obtaining those uniform conditions resulted in the selection of 3 animals per
559 genotype. Three biological replicates are accepted as a minimum number of animals
560 for mRNA expression analysis, as shown by recent published studies (Shi H. et. al,
561 2015; Yao D. W. et al., 2016 a, b; Weller et al., 2016). The expression of the gene in
562 both the udder and the liver was significantly influenced by the animals' genotype,
563 with ewes homozygous for the T allele showing significantly increased expression, by
564 several orders of magnitude, compared to ewes homozygous for the C allele. The
565 reasons why heterozygous mRNA expression in the udder exhibited a very high
566 standard deviation needs to be elucidated by further research.

567 To assess whether the observed genotype effects in the expression of the *ACAA2*
568 gene could be attributed to allelic expression imbalance (AEI), an AEI assay using
569 TaqMan probes was designed. The advantage of measuring AEI compared to total
570 transcript levels is the reduction of the confounding effect of trans-acting factors,
571 because the alleles are compared within and not across individuals (Bray et al., 2003;
572 Cowles et al., 2002; Forton et al., 2007). The results obtained are in support of
573 differential expression of the two alleles of the *ACAA2* gene due to a cis-acting
574 mechanism. Differential allelic expression has been found in a large proportion of
575 human (Bray et al., 2003; Yan et al., 2002), mouse (Campbell et al., 2008) and cattle
576 (Karim et al., 2011, Olbromski et al., 2013, Sasaki et al., 2013) genes and there is
577 growing evidence that polymorphisms in regulatory DNA and the resulting variability
578 in gene expression, can explain a significant proportion of disease susceptibility and

quantitative trait phenotypic variance (Bray et al., 2003, Khatib, 2007, Karim et al., 2011, Sasaki et al., 2013).

Consistent with other studies (Yan et al., 2002; Bray et al., 2003), the differential allelic expression of the *ACAA2* gene varied among individuals in post-mortem random samples, with an overall observed increase of T relative to C allele expression in the udder of heterozygous ewes close to 20%. Previous studies in cattle indicate that even small gene expression imbalances may result in large phenotypic variance of complex traits (Karim et al., 2011, Sasaki et al., 2013). Interestingly, mean AEI of the *ACAA2* gene was only significant in the udder but not in the liver. Such tissue specific AEI has also been reported for cattle genes (Olbromski et al., 2013, Chamberlain et al., 2015). This finding, together with the observed difference between mammary gland and liver in the average mRNA gene expression in TT relative to CC ewes (11.8 and 2.8 fold increase, respectively), suggest an organ specific differential expression. Preferential, or higher expression in organs related to a quantitative trait is an important criterion for the selection of functional candidate genes (Ron and Weller, 2007; Stickens et al., 2010).

Since no other polymorphisms have been detected in the mRNA sequence of the *ACAA2* gene, the differential mRNA expression of homozygotes and the observed AEI in the mammary gland could be attributed to the g.2982T>C SNP acting in cis to modulate gene transcription or mRNA survival (Pesole et al., 2001). Analysis of the region harbouring the SNP, using the MicroInspector program found differences in potential miRNA binding sites between the two alleles (Orford et al., 2012). However, since this in-silico analysis requires functional experimental support, the possibility of another cis acting regulatory polymorphism in linkage disequilibrium with the g.2982C>T SNP, e.g. in the promoter region of the gene, cannot be excluded.

Moreover, it is also likely that other trans-acting factors or mutations in other genes in linkage disequilibrium with *ACAA2,* found here to be associated with milk yield and protein content, may explain part of the QTL for milk, fat and protein yields observed in the region harbouring the *ACAA2* gene (Gutierrez- Gil et al., 2009).

ACAA2 is involved in mitochondrial fatty acid elongation and degradation (KEGG database: http://www.kegg.jp/), by catalyzing the last step of the respective β-oxidation pathway. In agreement with our data, although *ACAA2* is predominantly expressed in liver, it has also been reported to be expressed in the mammary gland (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ACAA2; Paten et al., 2015). In mammals, excess energy is stored primarily as triglycerides, which are mobilized when energy demands arise. During periods of underfeeding or in early lactation, ruminants cover their increased energy demands by mobilizing fat from the adipose tissue (Drackley, 1999). Fatty acids coming from triglycerides are taken up by the liver where they are either used as energy source or converted to ketone bodies that may be released into the blood and used as energy or substrates for de novo fatty acid synthesis in the mammary gland. In addition, it is estimated that gluconeogenesis is usually increased two- to three-fold during early lactation to meet the demands of the mammary gland for lactose and triglyceride synthesis (Drackley, 2000; Vernon, 2005). The increased energy needs for gluconeogenesis and triglyceride synthesis are met primarily by increased fatty acid oxidation. Additionally, a recent transcriptomics analysis of the ovine mammary gland has shown increased expression of genes of the b-oxidation pathway during late pregnancy (Paten et al., 2015). This contributes to the development of the udder that is crucial for subsequent lactation, as the number of active secretory cells primarily determines milk yield (Pollott GE, 2004). Therefore, although the mechanism by which the *ACAA2* gene could be linked to increased milk

yield and decreased protein percentage needs to be elucidated, increased *ACAA2* expression at the mRNA level is likely to result in higher levels of the enzyme and thus elevated amount of energy and carbon substrates for mammary development and lactation.

CONCLUSION

In the current study, we demonstrated that the variants in the 3'UTR of the *ACAA2* gene, which are associated with milk yield, protein percentage and fat yield, are differentially expressed in homozygous ewes of each allele and exhibit AEI within heterozygotes in a tissue specific manner, suggesting the existence of a cis-acting regulatory DNA mechanism. These findings support the hypothesis that the *ACAA2* gene is a functional candidate affecting dairy traits.

ACKNOWLEDGMENTS

This work was funded by the Cyprus Research Promotion Foundation, the European Structural Funds and the Cyprus University of Technology. G. Banos and A. Psifidi were supported by the BBSRC Institute Strategic Programme Grant (BB/J004235/1; UK) and G. Banos also by the Rural & Environment Science & Analytical Services Division of the Scottish Government. We thank the professional veterinarian John Pierroua for obtaining biopsies. We also thank the head of the sheep and goat farming sector of the Ministry of Agriculture, Mr. Paniko Christoforou, the head (Mr. Giorgo Aspromalli) and technical staff of the governmental farm (Orites) and the owners of the commercial farms for giving us access to their farms and help in sampling and collection of data.

654 **Table 1.** Predicted genotype means and standard errors of the genotypic classes CC,
655 CT and TT at the g.2982T>C *ACAA2* locus and significance of genotype contrasts for
656 each trait.

Trait	all lactations milk yield[1]	1st lactation milk yield[1]	milk protein percentage	milk fat percentage	milk protein yield[1]	milk fat yield[1]
	Genotyped means ± SE[2]					
CC	245.23 ± 4.44[a]	170.21 ± 5.28[a]	5.29 ± 0.02[a]	5.19 ± 0.05	8.78 ± 0.25	12.87 ± 0.26[a]
CT	260.71 ± 2.76[c]	193.85 ± 3.33[d]	5.23 ± 0.01[c]	5.20 ± 0.03	9.42 ± 0.16	13.62 ± 0.17[d]
TT	258.85 ± 3.88[b]	191.44 ± 4.77[c]	5.20 ± 0.02[c]	5.14 ± 0.04	9.23 ± 0.25	13.44 ± 0.23[b]

657 [1]Yields are in kg.
658 [2]Marginal genotype means (± standard error) predicted from the mixed model analyses
659 [a-e]Means within a column with two different superscripts differ as: [a,b]$P < 0.025$; [a,c]$P < 0.010$; [a,d]$P <$
660 0.001 after Holm-Bonferroni adjustment.
661

662 **Table 2.** SNP allelic effects and percentage of phenotypic variance explained by the
663 g.2982T>C SNP of the *ACAA2* gene.

Trait	all lactations milk yield	1st lactation milk yield	milk protein percentage	milk fat percentage	milk protein yield	milk fat yield
a[1] ± SE	6.81 ± 2.95	10.61 ± 3.56	-0.05 ± 0.02	-0.03 ± 0.03	0.23 ± 0.18	0.28 ± 0.17
P[2]	0.021*	0.003*	0.003*	0.370	0.200	0.100
d[3] ± SE	8.67 ± 3.53	13.02 ± 4.26	0.02 ± 0.02	0.04 ± 0.04	0.41 ± 0.21	0.46 ± 0.21
P[4]	0.014*	0.002*	0.41	0.322	0.048*	0.027*
% V_P due to SNP[5]	0.62%	2.25%	1.68%	0.33%	0.44%	0.41%

664 [1]a = additive effect; positive additive genetic effect (a > 0) indicates T allele increased the trait.
665 [2]*P* value for assessing the additive effect on the trait; *significant post Holm-Bonferroni adjustment for
666 three independent tests.
667 [3]d = dominance effect.
668 [4]*P* value for assessing the dominance effect on the trait *significant post Holm-Bonferroni adjustment
669 for three independent tests.
670 [5]Estimated using allele frequencies observed in sample (p = 0.46 for allele C and q = 0.54 for T).
671

672

673

REFERENCES

Andersson, L. 2001. Genetic dissection of phenotypic diversity in farm animals. Nature Reviews Genetics. 2:130-138.

AOAC International. 2005. official methods of analysis. 18th ed. 1st rev. AOAC international gaithersburg, MD.

Arranz, J. J. and B. Gutiérrez-Gil. 2012. Detection of QTL Underlying Milk Traits in Sheep: An Update. Milk Production - Advanced Genetic Traits, Cellular Mechanism, Animal Management and Health, Prof. Narongsak Chaiyabutr (Ed.), InTech, DOI: 10.5772/50762. Available from: Http://www.Intechopen.com/books/milk-Production-Advanced-Genetic-Traits-Cellular-Mechanism-Animal-Management-and-health/detection-of-Qtl-Underlying-Milk-Traits-in-Sheep-an-Update. INTECH Open Access Publisher

Bartlett, K.; Eaton, S. Mitochondrial β-oxidation. Eur. J. Biochem. 2004, 271, 462-469.

Bencini, R. and G. Pulina. 1997. The quality of sheep milk: A review. Animal Production Science. 37:485-504.

Bray, N. J., P. R. Buckland, M. J. Owen and M. C. O'Donovan. 2003. Cis-acting variation in the expression of a high proportion of genes in human brain. Hum. Genet. 113:149-153.

Chatziplis D. G.; Tzamaloukas, O.; Miltiadou, D.; Ligda, C.; Koumas, A.; Mavrogenis, A.P.; Georgoudis, A.; Papachristoforou, C. 2012. Evidence of major gene(s) affecting milk traits in the Chios sheep breed. Small Rum. Res. 105, 61–68.

Campbell, C. D., A. Kirby, J. Nemesh, M. J. Daly and J. N. Hirschhorn. 2008. A survey of allelic imbalance in F1 mice. Genome Res. 18:555-563.

Carta, A., S. Casu and S. Salaris. 2009. Invited review: Current state of genetic improvement in dairy sheep. J. Dairy Sci. 92:5814-5833.

Chamberlain, A. J., C. J. Vander Jagt, B. J. Hayes, M. Khansefid, L. C. Marett, C. A. Millen, T. T. Nguyen and M. E. Goddard. 2015. Extensive variation between tissues in allele specific expression in an outbred mammal. BMC Genomics. 16:1.

Chen, X., J. Weaver, B. A. Bove, L. A. Vanderveer, S. C. Weil, A. Miron, M. B. Daly and A. K. Godwin. 2008. Allelic imbalance in BRCA1 and BRCA2 gene expression is associated with an increased breast cancer risk. Hum. Mol. Genet. 17:1336-1348.

Clop, A., F. Marcq, H. Takeda, D. Pirottin, X. Tordoir, B. Bibé, J. Bouix, F. Caiment, J. Elsen and F. Eychenne. 2006. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat. Genet. 38:813-818.

713 Cochran, S. D., J. B. Cole, D. J. Null and P. J. Hansen. 2013. Discovery of single
714 nucleotide polymorphisms in candidate genes associated with fertility and
715 production traits in Holstein cattle. BMC Genetics. 14:1.

716 Cowles, C. R., J. N. Hirschhorn, D. Altshuler and E. S. Lander. 2002. Detection of
717 regulatory variation in mouse genes. Nat. Genet. 32:432-437.

718 De Rancourt, M., N. Fois, M. Lavín, E. Tchakérian and F. Vallerand. 2006.
719 Mediterranean sheep and goats production: An uncertain future. Small Ruminant
720 Research. 62:167-179.

721 Drackley, J. K. 1999. ADSA foundation scholar award: Biology of dairy cows during
722 the transition period: The final frontier? J. Dairy Sci. 82:2259-2273.

723 Drackley, J. 2000. Lipid metabolism. Farm Animal Metabolism and Nutrition. 97-
724 119.

725 Emery, R.S. 1988. Milk fat depression and the influence of diet on milk composition.
726 The Veterinary clinics of North America. Food animal practice 4 (2), pp. 289-
727 305

728 Forton, J. T., I. A. Udalova, S. Campino, K. A. Rockett, J. Hull and D. P.
729 Kwiatkowski. 2007. Localization of a long-range cis-regulatory element of IL13
730 by allelic transcript ratio mapping. Genome Res. 17:82-87.

731 Fuertes, J.; Gonzalo, C.; Carriedo, J.; San Primitivo, F. 1998. Parameters of test day
732 milk yield and milk components for dairy ewes. J. Dairy Sci. 81, 1300-1307.

733 Garcia-Gámez, E.; Gutiérrez-Gil, B.; Sahana, A. G.; Sanchez, J.P.; Bayon, Y.; Arranz,
734 J. J. 2012. GWA analysis for milk production traits in dairy sheep and genetic
735 support for a QTN influending milk protein percentage in the LALBA gene. PloS
736 One, 7(10), e47782.

737 Garcia-Gámez, E., B. Gutiérrez-Gil, A. Suarez-Vega, L. F. de la Fuente and J. J.
738 Arranz. 2013. Identification of quantitative trait loci underlying milk traits in
739 spanish dairy sheep using linkage plus combined linkage disequilibrium and
740 linkage analysis approaches. J. Dairy Sci. 96:6059-6069.

741 Gilmour, A. R., B. Gogel, B. Cullis, R. Thompson and D. Butler. 2009. ASReml user
742 guide release 3.0. VSN International Ltd, Hemel Hempstead, UK.

743 Gutiérrez-Gil, B., J. J. Arranz, R. Pong-Wong, E. García-Gámez, J. Kijas and P.
744 Wiener. 2014. Application of selection mapping to identify genomic regions
745 associated with dairy production in sheep. Plos One. 9:.

746 Gutiérrez-Gil, B., M. F. El-Zarei, L. Alvarez, Y. Bayón, L. F. De La Fuente, F. San
747 Primitivo and J. -. Arranz. 2009. Quantitative trait loci underlying milk
748 production traits in sheep. Anim. Genet. 40:423-434.

749 Holm S. 1979. A simple sequentially rejective multiple test procedure. Scandinavian
750 Journal of Statistics 6:65-70.

ICAR. 2014. ICAR (international committee for animal recording), 2014. section 2 ICAR rules, standards and guidelines for dairy production recording. approved by the general assembly held in berlin, germany, on may 2014.

Karim, L., H. Takeda, L. Lin, T. Druet, J. A. Arias, D. Baurain, N. Cambisano, S. R. Davis, F. Farnir and B. Grisart. 2011. Variants modulating the expression of a chromosome domain encompassing PLAG1 influence bovine stature. Nat. Genet. 43:405-413.

Khatib, H., V. Schutzkus, Y. Chang and G. Rosa. 2007. Pattern of expression of the uterine milk protein gene and its association with productive life in dairy cattle. J. Dairy Sci. 90:2427-2433.

Khatib, H. 2007. Is it genomic imprinting or preferential expression? Bioessays. 29:1022-1028.

Li HD (2008) I: Segregation, Cloning, SNP Detection and Its Association with Economic Traits of 5 Bovine Genes II: Quantitative Trait Loci Analysis of Meat Quality Traits in Pigs. Ph.D. thesis. Dissertation ID: 1647914. Available:http://www.dissertationtopic.net/doc/1647914.

Livak, K. J. 1999. Allelic discrimination using fluorogenic probes and the 5′ nuclease assay. Genet. Anal. : Biomol. Eng. 14:143-149.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 25:402-408.

Mavrogenis, A. and A. Constantinou. 1991. Selection Index and Expected Genetic Progress in Chios Sheep. Agricultural Research Institute, Ministry of Agriculture and Natural Resources.

Miltiadou, D., Orford M., Symeou S. and G. Banos. 2017. Identification of variation in the ovine prolactin gene with a cost effective sequence based typing (SBT) assay. J. Dairy Sci. 100 (2), pp. 1290-1294.

Muráni, E., S. Ponsuksili, T. Srikanchai, S. Maak and K. Wimmers. 2009. Expression of the porcine adrenergic receptor beta 2 gene in longissimus dorsi muscle is affected by cis-regulatory DNA variation. Anim. Genet. 40:80-89.

Olbromski, R., E. Siadkowska, B. Zelazowska and L. Zwierzchowski. 2013. Allelic gene expression imbalance of bovine IGF2, LEP and CCL2 genes in liver, kidney and pituitary. Mol. Biol. Rep. 40:1189-1200.

Orford, M., O. Tzamaloukas, C. Papachristoforou and D. Miltiadou. 2010. Technical note: A simplified PCR-based assay for the characterization of two prolactin variants that affect milk traits in sheep breeds. J. Dairy Sci. 93:5996-5999.

Orford, M., G. Hadjipavlou, O. Tzamaloukas, D. Chatziplis, A. Koumas, A. Mavrogenis, C. Papachristoforou and D. Miltiadou. 2012. A single nucleotide

790 polymorphism in the acetyl-coenzyme A acyltransferase 2 (ACAA2) gene is
791 associated with milk yield in chios sheep. J. Dairy Sci. 95:3419-3427.

792 Othmane M.H., Carriedo J.A., De la Fuente L.F., San Primitivio F. (2002): Factors
793 affecting test-day milk composition in dairy ewes, and relationships amongst
794 various milk components. J. Dairy Res., 69, 53–62.

795 Paten, A.M., J.E. Duncan, S.J. Pain, S.W. Peterson, P. R. Kenyon, H.T. Blair and P.K.
796 Dearden. 2015.Functional development of the adult ovine mammary gland—
797 insights from gene expression profiling. BMC Genom. 16:748

798 Pesole, G., F. Mignone, C. Gissi, G. Grillo, F. Licciulli and S. Liuni. 2001. Structural
799 and functional features of eukaryotic mRNA untranslated regions. Gene. 276:73-
800 81.

801 Pollott G.E. 2004. Deconstructing milk yield and composition during lactation using
802 biologically based lactation models. J Dairy Sci. Aug;87(8):2375-87.

803 Pulina G., Macciotta N., Nudda A. (2005): Reproductive performance and milk
804 production of Assaf sheep in an intensive management system. Ital. J. Anim. Sci.,
805 4, 5–14.

806 Ramón, M., A. Legarra, E. Ugarte, J. J. Garde and M. D. Pérez-Guzmán. 2010.
807 Economic weights for major milk constituents of manchega dairy ewes. J. Dairy
808 Sci. 93:3303-3309.

809 Ron, M. and J. Weller. 2007. From QTL to QTN identification in livestock–winning
810 by points rather than knock-out: A review. Anim. Genet. 38:429-439.

811 Sari, I. P., A. Rao, J. T. Smith, A. J. Tilbrook and I. J. Clarke. 2009. Effect of RF-
812 amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone
813 synthesis and secretion in ovine pituitary gonadotropes. Endocrinology.
814 150:5549-5556.

815 SAS. 2005. Statistical Analysis Systems User‘s Guide. Version 9.1.3. SAS Institute,
816 Inc., Cary, NC.

817 Sasaki, S., T. Ibi, T. Watanabe, T. Matsuhashi, S. Ikeda and Y. Sugimoto. 2013.
818 Variants in the 3' UTR of general transcription factor IIF, polypeptide 2 affect
819 female calving efficiency in japanese black cattle. BMC Genet. 14:.

820 Shi, H., J. Zhu, J. Luo, W. Cao, H. Shi, D. Yao, J. Li, Y. Sun, H. Xu, K. Yu and J. J.
821 Loor. 2015. Genes regulating lipid and protein metabolism are highly expressed
822 in mammary gland of lactating dairy goats. Funct. Integr. Genomics. 15:309-321.

823 Sodhi, S. S., M. Ghosh, K. D. Song, N. Sharma, J. H. Kim, N. E. Kim, S. J. Lee, C.
824 W. Kang, S. J. Oh and D. K. Jeong. 2014. An approach to identify SNPs in the
825 gene encoding acetyl-CoA acetyltransferase-2 (ACAT-2) and their proposed role
826 in metabolic processes in pig. PloS One. 9:e102432.

827 Stinckens, A., M. Schroyen, L. Peeters, S. Janssens and N. Buys. 2010. Quantitative
828 trait mutations in cattle, sheep and pigs: A review. Animal Science Reviews. 59.

Sugimoto, M., Y. Gotoh, T. Kawahara and Y. Sugimoto. 2015. Molecular effects of polymorphism in the 3'UTR of unc-5 homolog C associated with conception rate in holsteins. PloS One. 10:e0131283.

Vernon, R. G. 2005. Lipid metabolism during lactation: A review of adipose tissue-liver interactions and the development of fatty liver. J. Dairy Res. 72:460-469.

Weller, M. M. D. C. A., R. L. Albino, M. I. Marcondes, W. Silva, K. M. Daniels, M. M. Campos, M. S. Duarte, M. L. Mescouto, F. F. Silva and S. E. F. Guimarães. 2016. Effects of nutrient intake level on mammary parenchyma growth and gene expression in crossbred (holstein × gyr) prepubertal heifers. J. Dairy Sci. 99:9962-9973.

Xie, X., J. Lu, E. Kulbokas, T. R. Golub, V. Mootha, K. Lindblad-Toh, E. S. Lander and M. Kellis. 2005. Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature. 434:338-345.

Yan, H., W. Yuan, V. E. Velculescu, B. Vogelstein and K. W. Kinzler. 2002. Allelic variation in human gene expression. Science. 297:1143.

Yao, D. W., J. Luo, Q. Y. He, J. Li, H. Wang, H. B. Shi, H. F. Xu, M. Wang and J. J. Loor. 2016a. Characterization of the liver X receptor-dependent regulatory mechanism of goat stearoyl-coenzyme A desaturase 1 gene by linoleic acid. J. Dairy Sci. 99:3945-3957.

Yao, D. W., J. Luo, Q. Y. He, H. F. Xu, J. Li, H. B. Shi, H. Wang, Z. Chen and J. J. Loor. 2016b. Liver X receptor a promotes the synthesis of monounsaturated fatty acids in goat mammary epithelial cells via the control of stearoyl-coenzyme A desaturase 1 in an SREBP-1-dependent manner. J. Dairy Sci. 99:6391-6402.

FIGURE CAPTIONS

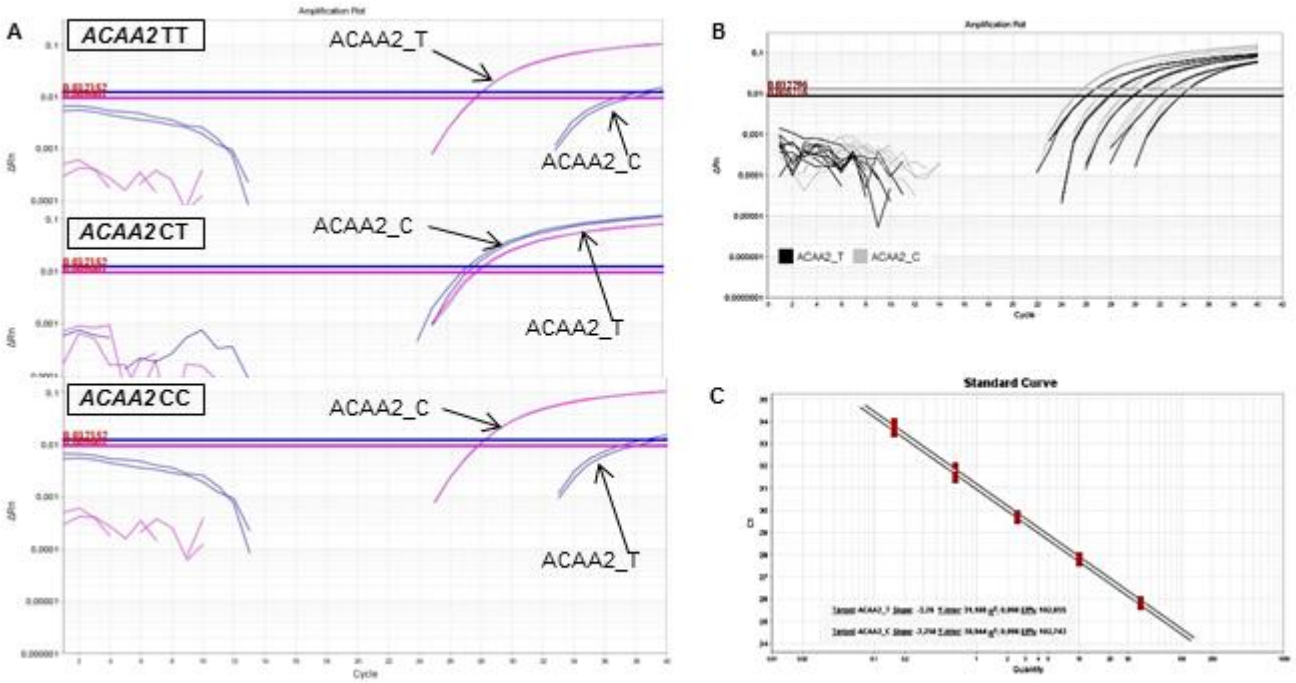
Figure 1. Allelic expression imbalance TaqMan Assay for the quantification of the *ACAA2* allele T:C transcription ratio. A. Amplification plots of TaqMan probe PCR from *ACAA2* TT (top), TC (middle) and CC (bottom) individuals. **B.** Amplification plot of serial dilution of standard gDNA pooled from three *ACAA2* TC ewes. **C.** Log_{10}Quantity and threshold cycle (Ct) relationship graph for T and C alleles. Red dots represent standard gDNA dilution series reactions. Linear standard curves for T and C alleles (targets), standard curve slope, Y-intersection, r^2 and efficiency values are shown.

Figure 2. Expression of the *ACAA2* gene in liver and udder. A. *ACAA2* expression in liver and udder samples (taken 42 ± 2 days after lambing) of CC, CT and TT first parity, 15 month old ewes that gave birth to two lambs each relative to the mean normalized expression in CC ewes. Expression was normalized by *β-actin* and *β-microglobulin* (B2M) expression in liver and udder, respectively. n=3 for all means except udder CT (n=2). **B.** Mean normalized expression of the *ACAA2* gene in *ex vivo* liver and udder samples from CC, CT and TT ewes, normalized by corresponding *b-actin* transcription and relative to mean normalized transcription in the liver of TT individuals. CC: n=1, CT: n=10 (udder) n=9 (liver), TT: n=4 (udder) n=5 (liver). Bars represent mean relative normalized transcription and standard error of the mean is shown. Means with different letters are significantly different ($P < 0.05$).

Figure 3. Sequencing of heterozygous samples from gDNA and cDNA from liver (A) and udder (B). The T/C SNP is shown in light blue shade. The peak heights of the T and C alleles are the same in gDNA, whereas the peak height of the T allele is higher compared to the C allele in cDNA of the same samples.

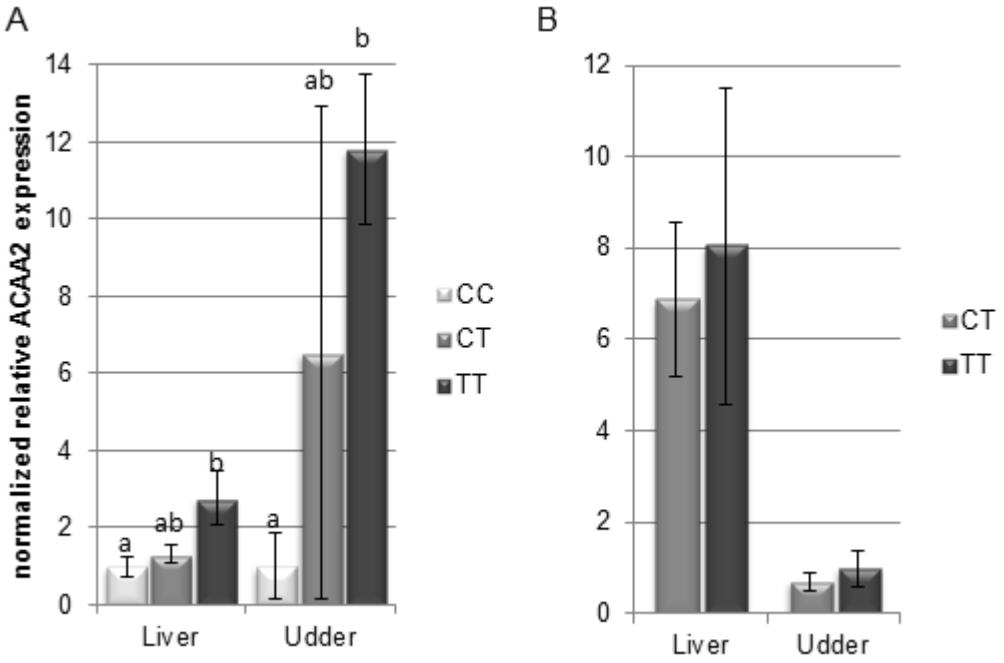
878 **Figure 4.** Allelic expression imbalance of the *ACAA2* gene. Graph shows TaqMan
879 assay corrected T:C ratios in the liver and udder of heterozygous ewes. Horizontal
880 lines represent mean corrected T:C allele in the cDNA from liver (mean=1.03, n=12)
881 and udder (mean=1.18, n=11) and in gDNA (mean=1, n=11) of *ACAA2* heterozygous
882 ewes. Open circles represent corrected T:C ratios for individual ewes. * $P < 0.05$, ** P
883 < 0.01.
884
885
886

887 Miltiadou et al. **Figure 1**



888

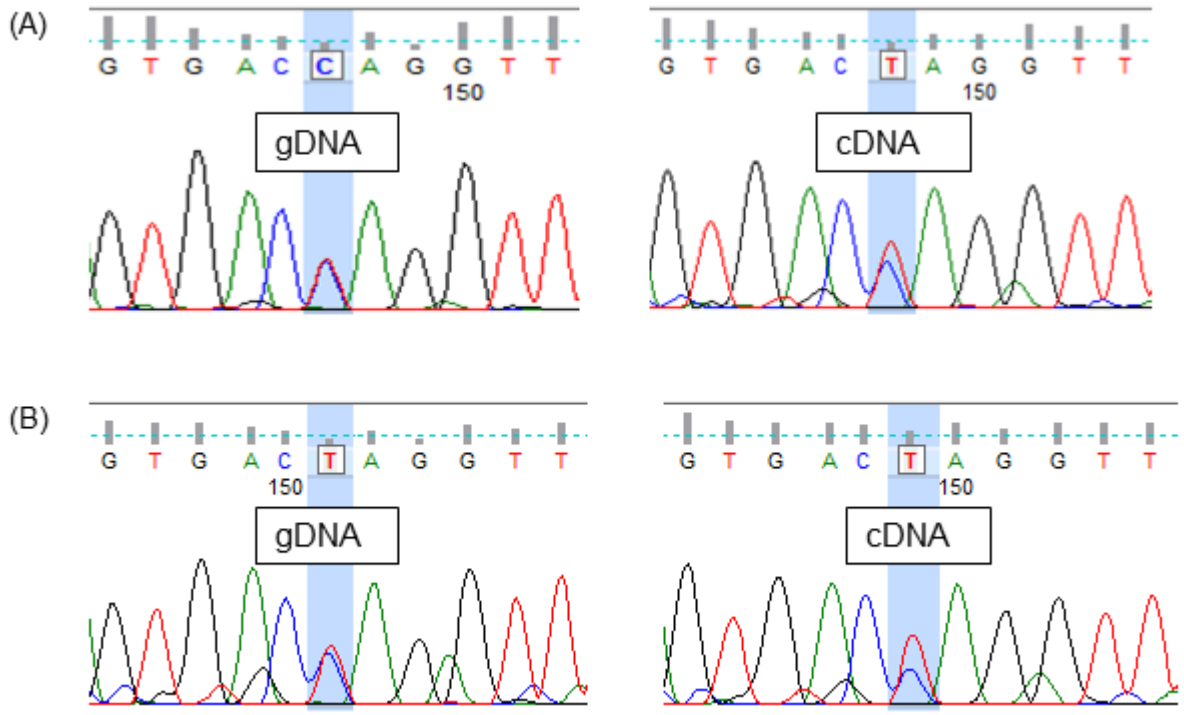
889 Miltiadou et al. **Figure 2**



890

891

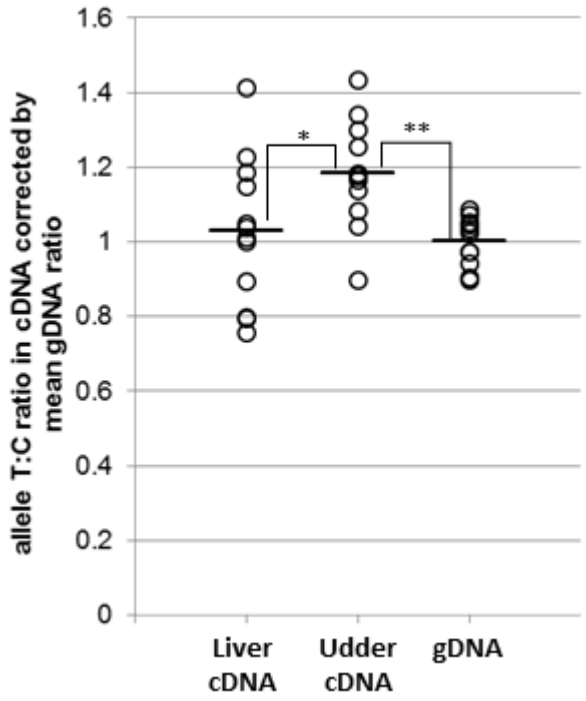
892 Miltiadou et al. **Figure 3**



893

894

895 Miltiadou et al. **Figure 4**

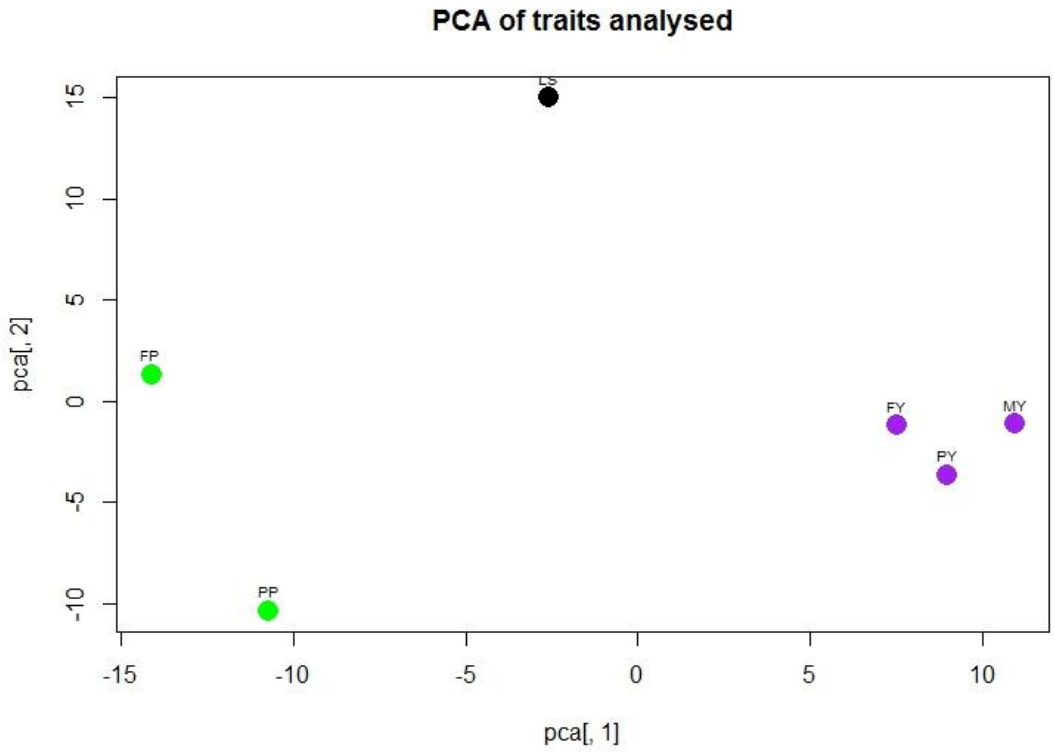


896

897

898 **Appendix**

899 **Supplementary Figure 1**: Principal Component Analysis of studied traits: milk yield
900 (MY), protein yield (PY), fat yield (FY), protein percentage (PP), fat percentage (FP)
901 and litter size (LS).



902

903 Supplementary Table 1

P values after Holm-Bonferroni adjustment		
Lowest *P* value	Second lowest *P* value	Third and later lowest P value
P = 0.0167	*P* = 0.025	*P* = 0.05

904